NOTE

Calcofluor Fluorescence Assay for Wort β-Glucan in a Microplate Format

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The level of β-glucan in worts produced from malted barley is a critical malting quality parameter. Measurement of wort β-glucan levels allows maltsters to determine whether production malts meet commercial specifications, helps brewers avoid production problems due to slow lautering, and enables barley breeders to develop lines with the potential to meet commercial needs. Many laboratories performing routine wort β-glucan analyses use flow injection analysis (FIA) methods or related segmented flow analysis (SFA) methods that measure the increase in fluorescence with Calcofluor binding to β-glucan. Such automated systems are attractive due to low per-sample reagent costs, relatively high sample throughput, and a minimal number of sample processing steps prior to automated analysis, limiting the need for manual operations. However, FIA or SFA methodology requires significant capital investment for instrument acquisition, discouraging its use outside dedicated quality assurance laboratories. Laboratories that routinely analyze relatively few samples often find kits for enzymatic or colorimetric analysis of β-glucans more attractive. Such kits require relatively simple laboratory instrumentation, reducing capital costs, but may involve multiple sample manipulations, increasing the level of technical support necessary to perform the analyses. Per-analysis reagent costs may also be greater for the kits. In this work, we have adapted the Calcofluor fluorescence method to a microplate reader to achieve a simple and cost-effective assay for wort β-glucan that avoids the acquisition costs of FIA or SFA instrumentation.

MATERIALS AND METHODS

Reagents were prepared according to ASBC Wort-18 specifications as modified in the Skalar SFA instrument manufacturer's instructions. Briefly, the differences from ASBC Wort-18 include use of a working Calcofluor concentration of 35 ppm in 0.01% Triton X-100/100 m*M* Tris-HCl, pH 8.0, rather than 25 ppm in 100 m*M* glycine, pH 9.0, and a β-glucan standard made to 400 rather than 300 ppm. Congress worts (ASBC 2004, Malt-4) were prepared from six ASBC malt checks chosen to represent low, intermediate, and high β-glucan levels. Congress worts from 57 malting barley lines selected to represent a range of values for various malting quality attributes commonly measured were also surveyed. The lines included two- and six-rowed barley from nurseries grown in Aberdeen, ID, Sidney, MT, Crookston, MN, and Morris, MN, and included named varieties and experimental lines. Other barley and malt analyses followed standard ASBC methodology.

Microplate β-glucan assays were conducted in flat-bottom, solid, black 96-well plates using a dual monochromator microplate fluorometer (Molecular Devices Spectramax Gemini XS). Each well contained a total assay volume of 200 µL. Routinely, a series of duplicate aliquots (0–10 µL) of a 400 ppm β-glucan standard was used to generate a standard curve up to 4.0 µg of β-glucan per well. Similarly, a series of duplicate aliquots of Congress wort (2–10 µL) were also delivered to appropriate wells generating a dilution series of each wort. Appropriate volumes of reagent grade water were added to each well to a total volume of 100 µL for standards and samples. When all wells had complete additions of wort, β-glucan standard, and water, the Calcofluor reagent was added using an eight-place electronic pipettor in a multidispense mode delivering 100-µL aliquots to each well. After the Calcofluor addition was complete, the plate was mixed on a microplate shaker and immediately read in the microplate fluorometer (Ex 365 nm, Em 425 nm, cutoff 420 nm). Relative fluorescence values for each well were exported to Excel for subsequent calculations.

Wort-dependent β-glucan signal quenching was determined across the 57 lines tested by constructing crossed-gradient matrix microplates in which dilution curves of tested worts and internal standards were constructed across the columns and rows, respectively, of 96-well microplates. Each well in columns 1/2, 3/4, 5/6, 7/8, 9/10, or 11/12 of the microplate received 0, 2.5, 5, 7.5, 10, or 12.5 µL of wort, respectively. Concurrently, a similar dilution series of internal standard β-glucan was generated across rows of the plate. That is, each well in row A, B, C, D, E, or F of the microplate received an addition of 0, 2.5, 5, 7.5, 10, or 12.5 µL of 400 ppm β-glucan standard resulting in 0, 1, 2, 3, 4, or 5 µg of β-glucan standard, respectively, in each well. This resulted in columns 1 and 2 containing a β-glucan standard curve (in 0 µL of wort) while, at the same time, row A contained a dilution series of 0–12.5 µL of wort. The standard curve generated from columns 1 and 2 was used to calculate the wort β-glucan concentration from the dilution series in row A. The remaining wells on the plate (columns 3–12 of rows B–F) contained β-glucan from a combination of both internal standard- and wort-derived β-glucan.

RESULTS AND DISCUSSION

Adapting the reagents used in common FIA or SFA of wort β-glucan to a microplate format has allowed generation of a linear standard curve (Fig. 1, inset) over a range of β-glucan concentrations. Standards up to 3 µg were fit using a linear regression. Standard curves using higher amounts (4–5 µg) (Fig. 2A, solid circles) can be fit using a second-order regression (EBC 2007, Analytica-EBC 8.13.2) to accommodate the nonlinearity found with higher standard amounts if an expanded range is desired. The amounts of β-glucan calculated for dilutions of various worts also shows a linear relationship (Fig. 1), with the regression slope

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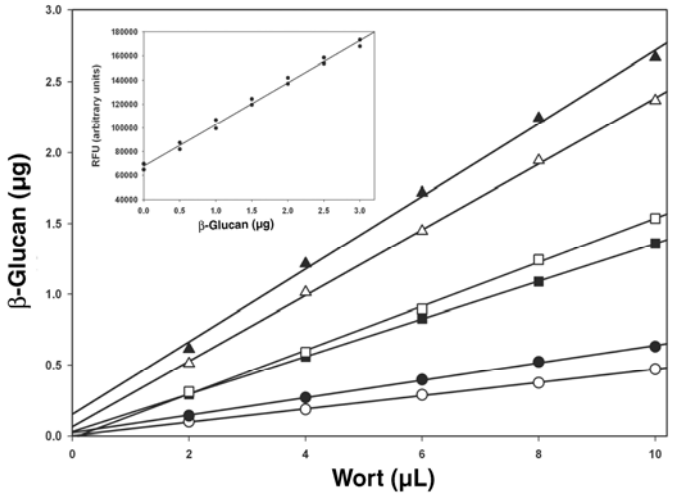

Fig. 1. Microplate β-glucan determination showing dilution series for six ASBC malt check samples. Inset shows a standard curve derived from duplicate assays. Malt check samples include Mal 06 1B (open circles, 46.8 ppm), Mal 06 2B (solid circles, 61.9 ppm), Mal 07 2B (solid squares, 133.1 ppm), Mal 06 1A (open squares, 155.2 ppm), Mal 05 4A (open triangles, 232.2 ppm), and Mal 06 3A (solid triangles, 256.9 ppm).

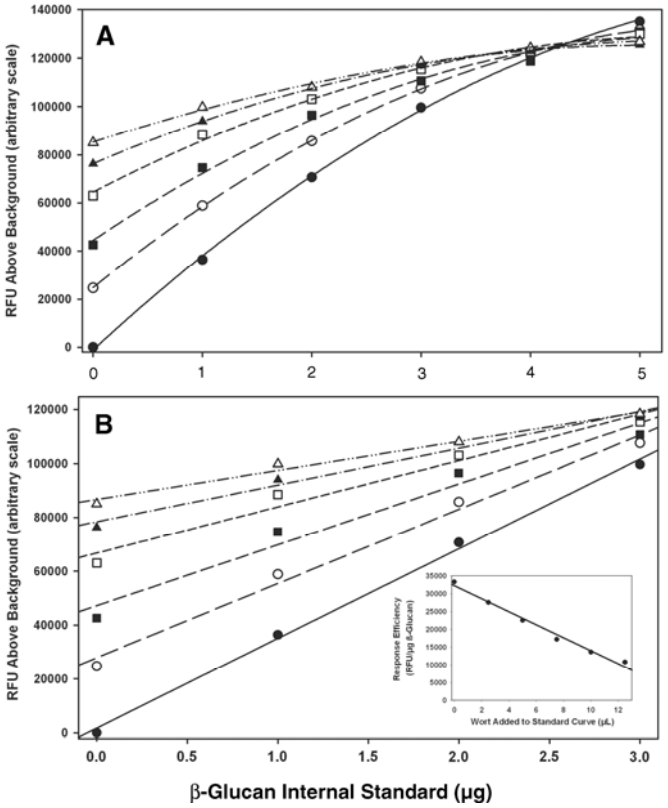

Fig. 2. Wort-dependent β-glucan signal reduction in a Congress wort made from Tradition malt (277 ppm β-glucan in wort). Standard curves of 0–5 µg of β-glucan were generated in the presence of 0–12.5 µL of wort (0 µL = solid circle, solid line; 2.5 µL = open circle, long dashed line; 5.0 µL = solid square, medium dashed line; 7.5 µL = open square, short dashed line; 10.0 µL = solid triangle, dot dash line; 12.5 µL = open triangle, dot dot dash line). **A,** Full response of matrix (0–5 µg of β-glucan and 0–12.5 µL of wort). **B,** Expanded view of initial portion of response with linear regression fits to data. All fits showed $r^2 > 0.97$. Slopes are efficiencies of Calcofluor response (relative fluorescence value [RFU]/µg β-glucan). Inset in B plots shows decline in assay efficiency as a function of volume of wort added.

(µg/µL) for the wort dilution series yielding the concentration of β-glucan in the wort tested. The ease of generating multiple dilutions in the microplate format and the ability to include the multiple dilutions on a single microplate makes it feasible to routinely analyze several dilutions of a sample to directly determine β-glucan concentration from the slope of the dilution series rather than relying on calculations from a single sample dilution where sampling errors might be magnified. The data in Fig. 1 show that linear slopes are readily determined for worts ranging in β-glucan concentration from <50 to >250 ppm. The assay can accommodate wort β-glucan concentrations >400 ppm (data not shown).

Results from the microplate β-glucan assay of six ASBC check malts shown in Fig. 1 are compared with results from concurrent analyses of the six worts by SFA methodology in Table I. This intralab comparison of the two analysis methods (SFA and microplate) applied to common worts allows a direct comparison of the two analytical methodologies. The microplate method yields similar magnitude concentration estimates for the low, intermediate, and high β-glucan samples, with comparable standard deviations for the differing analyses. Coefficients of variation for the ASBC check malts were ≈4–6% for the β-glucan check malts <100 ppm to <2% for the β-glucan worts >200 ppm. Variability among the analyses of the six check malts was low enough that samples were not only differentiated into low, intermediate, and high ranges but also were significantly differentiated from each other within each range (data not shown).

The microplate method yields lower estimates (significant at the 1% level by paired *t*-tests) of the β-glucan concentrations than the standard SFA method for the ASBC check malts (Table I). The larger sample of 57 worts from diverse barley lines had a strong correlation ($r^2 = 0.88$, slope = 1.02, intercept = 26 ppm) (data not shown) with the microplate measurements again significantly lower (1% level, paired *t*-test) than the SFA results. The regression analysis indicates that both the SFA and microplate methods respond comparably to incremental differences in wort β-glucan (regression slope = 1.02), and that the SFA method overestimates the wort concentration determined by the microplate method by ≈20–30 ppm. The Cereal Crops Research Unit comparative intralab standard deviations of either the microplate or standard method (measuring the same worts using shared reagents) are much lower than the interlab standard deviations in reported analyses from different laboratories participating in the malt check service collaborative study, suggesting that the mashing conditions or other parameters are significant sources of β-glucan analytical variability.

One of the advantages of the microplate format assay system is the ability to easily include a relatively large number of treatments in a single experiment. As described above, it is straightforward to use multiple sample dilutions and not be limited to a single dilution in the analysis. However, other manipulations are also possible, such as adding β-glucan internal standards to the wort dilutions. Such additions of authentic β-glucan to worts allows measurement of the recovery of the standard additions, and thus measurement of assay efficiency. Results from such an experiment in which orthogonal gradients in authentic (exogenous) β-glucan and wort-derived (endogenous) β-glucan were generated in the microplate are shown in Fig. 2. The data shown present the matrix as a series of exogenous β-glucan standard curves constructed in varying amounts of wort. In the absence of signal quenching, the family of curves would have been seen as a series of parallel lines, with the wort-containing curves offset by the amount of β-glucan in the worts added. However, Fig. 2A shows that this result is not seen. Instead, the series of curves converge. Indeed, at the highest concentration of exogenous β-glucan standard, addition of wort decreases the fluorescent signal below that of the β-glucan standard alone. One potential explanation of this phenomenon is that a component of the wort causes a loss in fluorescent signal, consistent with the findings of Izawa et al (1996).

TABLE I
Wort β-Glucan Measurements on Six ASBC Malt Check Collaborative Samples[a]

ASBC Malt Check Collaborative Sample ID	ASBC Malt Check Collaborative Results[b]	CCRU Contemporary SFA Results	CCRU Comparative SFA Results[c]	CCRU Comparative Microplate Results[d]
2006 Mal 2B	68.2 ± 15.5 (18)	57	75.4 ± 1.3	61.6 ± 2.7
2006 Mal 1B	66.3 ± 21.9 (20)	70	66.0 ± 2.5	46.7 ± 2.7
2006 Mal 1A	164.2 ± 49.6 (20)	153	184.6 ± 2.6	150.9 ± 4.7
2007 Mal 2B	155.3 ± 35.6 (21)	160	154.7 ± 0.5	132.3 ± 3.8
2005 Mal 4A	258.0 ± 84.4 (17)	219	275.7 ± 2.1	228.4 ± 3.7
2006 Mal 3A	294.4 ± 61.0 (17)	328	310.7 ± 2.6	248.8 ± 4.5

[a] For malt check program details and collaborative results, see www.asbcnet.org/checksample/default.htm. Cereal Crops Research Unit (CCRU) contemporary segmented flow analysis (SFA) results were generated when each malt check sample was originally distributed. CCRU SFA and microplate comparative results were generated on common Congress worts from retained check malt samples mashed in March 2008. Standard deviations reported for malt check collaborative results are interlab standard deviations, but standard deviations reported for CCRU comparative results are intralab standard deviations. Values reported are mean ± standard deviation.
[b] Number of collaborating laboratories in parentheses.
[c] Four analytical replicate analyses of common Congress worts.
[d] Eight analytical replicate analyses of common Congress worts.

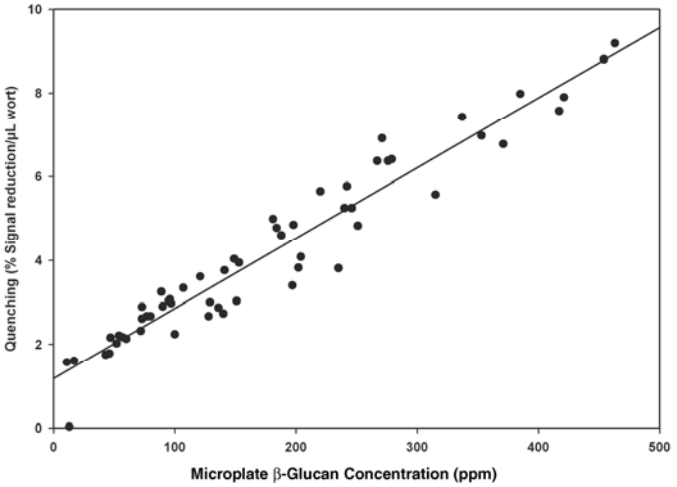

Fig. 3. Loss of calcofluor/β-glucan fluorescence with increasing wort β-glucan. Worts from 57 diverse barley malts were analyzed for signal loss using a crossed-gradient matrix of wort dilutions by internal standard β-glucan additions calculated as shown in Fig. 2. Degree of signal loss is plotted as a function of calculated wort β-glucan levels, with a linear correlation coefficient of $r^2 = 0.93$.

Estimating the wort response slope by fitting linear regressions to the initial portion of the dose curves (Fig. 2B) shows that increasing amounts of wort decrease the detection efficiency (slope) in a roughly linear fashion (Fig. 2B, inset). An estimate of the degree of signal loss can be calculated from this analysis. Estimates of the loss in fluorescent signal due to wort addition in the 57 worts tested found that the loss was ≈1.5–9%, and was strongly correlated with wort β-glucan levels (Fig. 3). Signal reduction was also correlated but to a much lesser extent ($r^2 = 0.22$) with additional malting quality parameters such as α-amylase activity and Kolbach index (data not shown). Although the decreases in response slope (assay efficiency) indicating wort-dependent signal loss are most apparent at higher concentrations, it should be noted that the reduction in signal is measurable at low levels of β-glucan and is not solely a phenomenon of high β-glucan levels. Whether the signal loss observed is due to photochemical quenching or to inner filter effects (Puchalski et al 1991) is unclear, as is the identity of the materials that may be responsible for the signal reduction. However, the results in this study confirm the earlier report by Izawa et al (1996) describing a loss of Calcofluor or β-glucan fluorescence intensity due to one or more wort components.

Because signal loss is present to some extent in essentially all samples tested, most estimates of β-glucan levels are likely underestimates, with greater underestimation seen in samples with higher β-glucan levels. Nonetheless, if greater accuracy in β-glucan measurements is desired, it is possible to measure and correct for the degree of signal reduction using internal standard additions.

An earlier report of a different implementation of a microplate Calcofluor β-glucan method (Riis 2005) also found the microplate assay generally useful, citing a similar slope (1.05 vs. 1.02 in this study), albeit with a somewhat lower intercept (3.8 vs. 26 ppm) for a comparison of FIA and microplate methods. However, Riis commented on difficulties in pipetting 20-μL aliquots of Congress wort reproducibly, apparently due to problems with wort viscosity and adherence to pipette tips. As a result, Riis recommended increasing sample sizes, using 50-μL samples in 48-well plates (total well volume 1,000 μL). In this study, we encountered no difficulties in pipetting even smaller volumes of Congress worts accurately and reproducibly (Fig. 1) using high-quality pipettes and pipette tips and careful pipetting techniques. Should reproducibility, or other issues related to manual pipetting, prove to be problematic, the microplate Calcofluor methods could be readily adapted to use of simple plate-filling devices, more complex diluter or dispenser instrumentation, or full robotic automation that would alleviate manual sample handling issues.

Another advantage to a microplate format assay over standard FIA or SFA implementation derives from the low sample requirement. Although standard Congress mashing protocols provide relatively large volumes of wort, newer alternative mashing protocols adapted for limited-availability malts used in research programs (Schmitt et al 2006) generate smaller volume worts than can be analyzed by traditional FIA or SFA methods. Other wort β-glucan methods such as the enzymatic (EBC 2007, Analytica-EBC 8.13.1) or colorimetric (EBC 2007, Analytica-EBC 4.16.3) methods, using kits offered by Megazyme (Bray, Ireland, www.megazyme.com) or Novabiotec, Dr. Fechter GmbH (Berlin, www.novabiotec.de), respectively, may also be adaptable to a microplate implementation for analysis of limited availability samples. However, a microplate Calcofluor method offers benefits due to simplicity of the assay and the cost and availability of reagents.

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